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Title: Impact of high-pressure carbon dioxide on polyphenoloxidase activity and stability of fresh apple juice

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Abstract: Freshly-extracted apple juice was exposed to high pressure carbon dioxide (HP-CO2) treatment at 20, 35 and 45 °C at different pressure conditions (6.0, 12.0 and 18.0 MPa) for up to 30 min. Samples were analysed for residual enzymatic activity. The time needed for 90% enzyme inactivation (Dp) decreased when CO2 pressure increased, while the CO2 pressure sensitivity of the enzyme (zp) showed no variation with temperature. The HP-CO2 treatment at 12 MPa and 35 °C allowed the minimum residual enzyme activity (20%) to be reached in 10 min. Samples treated under these conditions showed lower polyphenoloxidase activity and higher microbial stability than untreated apple juice while presenting a sensory fresh-likelihood higher than thermally pasteurized apple juice.

Dear Editor,

We considered further comments and suggestions and modified the manuscript accordingly. We are thus sending you the revised work.

Kind regards,

Stella Plazzotta

***Detailed Response to Reviewers**

Answer to Editor's comments:

Reference style: all authors (up to 6) should be given at the first citation of a publication (and in the subsequent citations 1st author et al, for more than 2 authors); if more than 6: 1st author et al also at first citation; in the reference list, according to APA "give surnames and initials for up to and including seven authors. When authors number eight or more, include the first six authors' names, then insert three ellipsis points, and add the last author's name".
References were modified according to Editor's suggestion (lines 50, 52, 60, 62, 333, 401).

L167-168: Two experiments for each treatment but how many juice batches? How many replicates for the juice production?
Requested information was added in lines 79-83 and 174-175.

Express enzyme activity in katals
Enzyme activity was expressed in katals as requested (lines 141-147, 441).

As Figures 5 and 6 are all related to sensory analysis could you please group them in Figure 5 A and B
Figures 5 and 6 were grouped as suggested (lines 346, 349, 357)

***Highlights (for review)**

- HP-CO_2 treatment promotes partial inactivation of polyphenoloxidase in apple juice
- HP-CO_2 treatment increases microbial stability of apple juice
- HP-CO_2 treatment does not impair apple juice fresh-likelihood

Impact of high-pressure carbon dioxide on polyphenoloxidase activity and stability of fresh apple juice

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Highlights

HP-CO_2 treatment promotes partial inactivation of polyphenoloxidase in apple juice

HP-CO_2 treatment increases microbial stability of apple juice

HP-CO_2 treatment does not impair apple juice fresh-likelihood

Abstract

Freshly-extracted apple juice was exposed to high pressure carbon dioxide (HP-CO_2) treatment at 20, 35 and 45 °C at different pressure conditions (6.0, 12.0 and 18.0 MPa) for up to 30 min. Samples were analysed for residual enzymatic activity. The time needed for 90% enzyme inactivation (D_p) decreased when CO_2 pressure increased, while the CO_2 pressure sensitivity of the enzyme (z_p) showed no variation with temperature. The HP-CO_2 treatment at 12 MPa and 35 °C allowed the minimum residual enzyme activity (20%) to be reached in 10 min. Samples treated under these conditions showed lower polyphenoloxidase activity and higher microbial stability than

untreated apple juice while presenting a sensory fresh-likelihood higher than thermally pasteurized apple juice.

Introduction

Consumption of unprocessed fruit juices has substantially risen over the last few years, mostly due to the increasing demand for good nutritional quality foods with fresh-like characteristics (Beuchat, 1996; Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny & Martín-Belloso, 2009). As a consequence of inappropriate manipulation and storage, both spoilage and pathogenic microorganisms can grow, leading to hygienic and quality issues. Enzymatic activity can also contribute to quality depletion, along with physical and chemical changes during the storage (Raybaudi-Massilia et al., 2009). To guarantee product safety and provide an adequate shelf-life, unpasteurized juices are generally distributed under refrigerated conditions. They are traditionally obtained by a combination of formulation strategies such as water activity reduction, nutrient restriction, acidification as well as use of antimicrobial additives (Davidson, 2001). These preservation strategies hardly fit with the current demand for fresh-like juices that are free from additives, generating the need for developing novel non-thermal treatments for juice stabilization.

High pressure carbon dioxide (HP-CO_2) has been reported as a promising non-thermal technology for the stabilization of fresh products. During the treatment, food is in contact with pressurised CO_2 at temperature/pressure conditions that may be below or above the critical point (31.1 °C, 7.38 MPa). Typical CO_2 pressure is generally within 4 and 30 MPa, rarely exceeding 50 MPa. Temperature is generally between 20 and 50 °C, low enough to maintain the freshlikelihood of treated products (Manzocco et al., 2016).

Significant lethal effects of HP-CO_2 on different microorganisms have been demonstrated in fruit juices (Spilimbergo & Bertucco, 2003; Damar & Balaban, 2006; Ferrentino, Bruno, Ferrari, Poletto & Balaban, 2009; Xu, Zhang, Wang, Bi, Buckow & Liao, 2011). In particular, the technology is known to promote up to 5 Log reductions in microbial counts, approaching those required for

pasteurization (Kincal, Hill, Balaban, Portier, Wei & Marshall, 2005; Ferrentino & Spilimbergo, 2011). The germicidal activity of HP-CO_2 is due to the combination of temperature, pressure and specific effects of HP-CO_2. The treatment is associated with extracellular and intracellular acidification, destabilization of membranes and denaturation of microbial enzymes (Jones & Greenfield, 1982; Hutkins & Nannen, 1993; Bothun, 2004; Bothun, Knutson, Strobel & Nokes, 2005). More controversial is the effect of HP-CO_2 in inactivating fruit enzymes leading to juice quality decay. For instance, inactivation of polyphenoloxidase responsible for browning of fruit juices, depends on the nature of the enzyme and is strongly affected by CO_2 pressure, temperature and treatment time (Gui, Chen, Wu, Wang, Liao & Hu, 2006; Liao, Zhang, Bei, Hu & Wu, 2009; Zhou, Zhang, Hu, Liao & He, 2009; Spilimbergo, Komes, Vojvodic, Levaj & Ferrentino, 2013). The mechanisms involved in enzyme inactivation by HP-CO_2 include pH lowering (Balaban, Arreola, Marshall, Peplow, Wei & Cornell, 1991) and changes in the conformation of the secondary structure of the enzyme (Chen, Balaban, Wei, Marshall & Hsu, 1992; Manzocco et al., 2016).

Based on these considerations, the present paper was addressed to investigate the impact of HP-CO_2 treatment on polyphenoloxidase activity and stability of fresh apple juice intended for refrigerated storage. To this aim, apple juice was exposed to HP-CO_2 treatments in a wide range of pressure, temperature and treatment time conditions. Apple juice was then submitted to the HP-CO_2 treatment leading to the minimum polyphenoloxidase activity at the mildest pressure/temperature combination and stored at 4 °C for up to 15 days. HP-CO_2 treated apple juice was monitored during storage for residual polyphenoloxidase activity, colour, microbial counts and sensory attributes. To verify the potential applicability of HP-CO_2 technology to produce fresh apple juice, data were compared to those relevant to an untreated apple juice. An apple juice submitted to conventional thermal pasteurization was also considered as additional control.

2. Material and methods

78 *2.1 Apple juice extract*

79 A 10 kg batch of fresh apples "Golden delicious" were purchased at the local market and stored at 4
80 °C overnight. When the experiments were performed, apples had a dry matter content of 164.7 ± 1.6
81 g/kg, a soluble solid content of 13.3 ± 0.2 °Brix, a pH of 4.2 ± 0.2 and a titratable acidity of
82 4.6 ± 0.3 g/kg. Apple juice was prepared fresh for every trial from the same batch of fruits, to
83 minimize sample variability. The juice was obtained by using a domestic juicer (Moulinex, mod.
84 Vitae JU2000, Milan, Italy), filtered through two layers of cloth filter and centrifuged at 5000 g for
85 5 min at 4 °C (Beckman, Avanti^{TM} J-25, High performance centrifuge, Brea, USA). The supernatant
86 was filtered again through two layers of cloth filter and the resulting clear juice was immediately
87 treated.

88

89 *2.2 High-pressure CO_2 treatments*

90 HP-CO_2 inactivation process was carried out in a double-batch apparatus. The system consists of
91 two identical stainless steel cylinders with a screwed cap and an internal volume of 150 mL,
92 connected in parallel. Each reactor was connected to an on-off valve that can be used to
93 depressurise it independently from the other. The two reactors were submerged in a thermostatic
94 water bath (CB 8-30e, Heto, Allerød, Denmark). For more details, please refer to Manzocco et al.
95 (2016). Before starting the pressurisation, the temperature of the sample was allowed to reach
96 equilibrium. The time needed to reach the desired temperature (20, 35 or 45 °C) and the
97 pressurisation time were lower than 3 min. After reaching the desired pressure (6, 12 and 18 MPa),
98 the pump was switched off and valves connected to each vessel were tightly closed. After
99 increasing treatment time up to 30 min, vessels were depressurised. In all experiments,
100 depressurisation was completed within 10 min and the outlet flow was controlled using a digital
101 flowmeter (PFM 750, SMC Italia S.p.A., Milan, Italy). Control samples were prepared by treating
102 the apple juice in the vessels at atmospheric pressure (0.1 MPa) and thus at CO_2 partial pressure
103 equal to 0.0039 MPa.

104

105 *2.3 Thermal treatment*

106 Aliquots of 100 mL of apple juice were placed in plastic pouches (PA/PE, 20 x 28 cm, Savonitti,
107 Codroipo, Italy). A thin layer of sample was obtained, being the maximum thickness of the filled
108 pouches lower than 0.5 cm. Pouches were heated in a water bath (IKA-Werke, Staufen, Germany)
109 at 71.1 °C for 6 s (FDA, 2004). After thermal treatment, samples were quickly cooled under
110 running water at room temperature.

111

112 *2.4 Apple juice storage*

113 Aliquots of 10 mL of apple juice were introduced in Eppendorf® vials of 10 mL capacity and
114 stored for up to 15 days at 4 °C in a refrigerated cell. At increasing time during storage, samples
115 were removed from the refrigerator, equilibrated at 22 °C and submitted to the analysis.

116

117 *2.5 Apple physical-chemical parameters*

118 Soluble solid content (SSC) was measured using a table refractometer (Unirefrax, Bertuzzi, Milan,
119 Italy) calibrated with distilled water.

120 Dry matter content of apple samples was determined gravimetrically by recording difference in
121 weight before and after drying at 70 °C, until a constant weight was achieved (M.U.A.C.V., 1989).

122 Titratable acidity was determined by titration with NaOH 0.1 mol/L and phenolphthalein as
123 indicator (Sigma-Aldrich, Milan, Italy), accordingly to the official M.U.A.C.V. method (1989) and
124 expressed as g of acids/kg of fresh product.

125 Analyses of SSC and TA were carried out on the solution obtained after homogenization (Polyton,
126 Kinematica, Luzern, Switzerland) and filtration of apple cubes through filter paper (Whatman #1,
127 Whatman International Ltd, Maidstone, UK).

128

129 *2.6 Temperature, pH*

During HP-CO_2 treatments and thermal pasteurization, temperature was measured by a thermocouple probe (Hanna Instruments, Tersid s.r.l., Milan, Italy); pH was assessed using a pH-meter (Mettler Toledo 355, Lou Analyzer, Halstead, England).

2.7 Polyphenoloxidase activity

The polyphenoloxidase activity was assayed spectrophotometrically (Shimadzu UV-2501PC, UV-Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 25 °C according to the methodology of Kahn (1995). The reaction was started by the addition of 500 µL of apple juice to 2 mL of 0.1 mol/L potassium phosphate buffer pH 7 and 1.5 10^{-3} mol/L L-Dopa (Carlo Erba, Milan, Italy). The absorbance at 420 nm was monitored each minute for 10 min. The changes in absorbance per min were calculated by linear regression, applying the pseudo zero order kinetic model. The eventual final stationary phase was excluded from regression data. The slope of the very first linear part of the reaction curve was used to determine polyphenoloxidase specific activity. The latter was defined as the amount of enzyme that produced 1 μmol of quinone per second (μkatal) (Lee et al., 2010). The average polyphenoloxidase activity in untreated juice was found to be 0.047 μkatals. Polyphenoloxidase residual activity (*RA*%) upon treatments was calculated as the percentage ratio between enzymatic activity of the treated sample and that of the untreated one (de la Rosa et al., 2011; Niu et al., 2010; Xu et al., 2011).

2.8 Browning

Browning was assessed spectrophotometrically (Shimadzu UV-2501PC, UV-Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) measuring absorbance values at 420 nm and 25 °C of apple juice samples, diluted with water to obtain absorbance signals within the scale.

2.9 Microbiological analyses

For microbiological analyses, appropriate aliquots (0.1 or 1 mL) of properly diluted apple juice were spread on agar plates. Plate Count Agar (Oxoid, Milan, Italy) and Man Ragosa Sharpe (MRS) (Oxoid, Milan, Italy) were used for enumeration of mesophilic and lactic acid bacteria respectively, and plates were incubated for 48 h at 37 °C. Oxytracycline-Glucose-Yeast Extract (OGYE) agar (Oxoid, Milan, Italy) was used for enumeration of yeasts and moulds, and plates were incubated for 72 h at 28 °C.

2.10 Sensory analysis and off-odour perception

A panel of 20 Italian assessors, not trained but expert in method, was selected. For sensory testing, 10 mL apple juice was served in odourless plastic glasses at 6 °C. Water was used between samples for mouth rinsing. Samples were indicated by a three-digit code and served the panel paired with a just prepared control sample, identified as "reference". The judges were asked to evaluate sample colour, apple flavour, cooked taste and acidity assigning each descriptor a score on a 9-point scale anchored with "reference" at point 5. Judges were also asked to indicate the possible perception of off-flavours. Off-flavour perception data were expressed as percentage of judges that identified the defect as respect to the reference. Sensory analysis requiring juice drinking was only carried out until all samples had total viable count lower than 5 Log CFU/g.

2.11 Data analysis

Data reported in this work are expressed as mean ± S.D of at least three measurements carried out on two experiments replicated on different juice extraction batches.

Apparent inactivation rate constants of polyphenoloxidase were analysed by using a conventional first-order equation:

$$\frac{d(RA\%)}{dt} = -k \cdot (RA\%) \qquad (1)$$

Where *RA%* is the polyphenoloxidase residual activity in the juice at time *t* (min) and *k* is the inactivation rate constant (min^{-1}). The value of *k* was obtained as the slope of the regression of the decimal logarithm of *RA%* vs. *t*.

The eventual final stationary phase was excluded from regression data.

The value of *RA%* after 30 min was taken as an indicator of the minimum *RA%* achievable by the treatment.

The kinetic parameter D_P was obtained using procedures analogous to that employed in thermal death time studies. In particular, D_P is the decimal reduction time, i.e. the treatment time needed for 90% enzyme activity reduction at a given pressure and temperature. D_P was computed as the negative reciprocal of *k*.

The pressure increase needed for a 90% reduction of the D_P value was computed as z_p (MPa). The value of z_p was obtained by regressing the decimal logarithm of D_P versus pressure (*P*):

$$logD_p = -\frac{P}{z_P} \qquad (2)$$

The z_P was then derived as the negative reciprocal slope of the regression line.

The pressure dependence of the inactivation rate constants (*k*) was expressed by the activation volume ($\Delta V^{\neq}$, cm^3/mol), according to the Eyring equation (Weemaes, Ludikhuyze, Van den Broeck & Hendrickx 1998):

$$lnk = lnk_{atm} - \frac{\Delta V^{\neq}}{RT} \cdot (P - P_{atm}) \qquad (3)$$

where *P* is pressure (MPa), k_{atm} is the inactivation rate constant at ambient pressure P_{atm} (0.1 MPa), *R* is the gas constant (8.31 cm^3 MPa K^{-1} mol^{-1}) and *T* is temperature (K). $\Delta V^{\neq}$ was estimated from the slope of the line obtained by the regression of the natural logarithm of *k* vs. *P*.

Goodness-of-fit was evaluated by means of the determination coefficients (R^2). Analysis of variance (ANOVA and Tuckey test) were accomplished using the v. 3.1.1 of R software (The R foundation for statistical computing), to determine the significance at a 95% level.

3. Results and discussion

3.1. Effect of high pressure carbon dioxide on apple juice polyphenoloxidase activity

Figure 1 shows the residual polyphenoloxidase activity of apple juice as a function of treatment time at different pressures at 20, 35 and 45 °C.

Control apple juice treated for increasing time at 20 °C under environmental conditions (0.1 MPa CO_2 pressure) showed a significant decrease in polyphenoloxidase activity. According to Le Bourvellec, Le Quéré, Sanoner, Drilleau & Guyot (2004), this effect is probably due to the formation of chemically oxidised polyphenols with anti-enzymatic activity upon contact of apple derivatives with oxygen. Exposure of apple juice to increasing CO_2 pressure resulted in progressively higher enzyme inactivation (Figure 1a). However, even applying 18 MPa for 30 min, the complete inactivation was not achieved. When apple juice was exposed to HP-CO_2 at 35 °C, polyphenoloxidase inactivation was more intense (Figure 1b). For instance, the minimum residual activity was reached upon few min of exposure to 18 MPa CO_2. The effect of temperature on enzyme inactivation by HP-CO_2 was further confirmed by additional trials carried out at 45 °C (Figure 1c). In accordance with evidences from other Authors (Vamos-Vigyazo, 1981; Gui et al., 2007), these data demonstrate the existence of a negative relation between polyphenoloxidase activity and the increase in both CO_2 pressure and temperature, at least under the experimental conditions here tested.

Data shown in Figure 1 were analysed considering the minimum residual activity achievable by each treatment (Table 1). Due to the monotonic decrease of residual activity curves (Figure 1), the value after 30 min of juice treatment was taken as an indicator of the residual activity achievable at each temperature/pressure combination (Table 1). In particular, under the most intense CO_2 treatment conditions (18 MPa, 45 °C), a minimum residual activity (*RA*%) of 20% was still observed. It can be hypothesised that more intense treatments than those here performed are needed to reach complete inactivation. To this regard, contradictory information is reported in the literature.

In particular, Xu et al. (2011) found that polyphenoloxidase was completely inactivated by a treatment carried out at 22 MPa and 60 °C for 10 min. A similar effect was also observed by Niu et al. (2010) in apple slices treated at 20 MPa and 25 °C for 20 min. However, other authors reported that even applying CO_2 at 60 MPa and 55 °C for 60 min, a 40% minimum residual activity of polyphenoloxidase was still present (Gui et al., 2007). These different inactivation degrees can be attributed to many factors, including not only operative conditions, but also apple cultivar and derivative as well as equipment layout and operative parameters (Yemenicioğlu, Özkan, Cemeroğlu, Mehmet & Yemeniciog, 1997; Weemaes et al., 1998; Buckow, Weiss & Knorr, 2009; Xu et al., 2011).

The residual activity of polyphenoloxidase was regressed as a function of treatment time in the initial linear part of the curve (Figure 1) to obtain rate constants (k) of polyphenoloxidase inactivation (Table 1). The latter were then used to calculate D_p values using procedures analogous to that employed in thermal death time studies (Table 1). In particular, D_p was defined as the treatment time needed for 90% enzyme activity reduction at a given pressure. The treatment at 6 MPa and 20 °C led to a tenfold decrease of activity in *circa* 48 min. At the same temperature, this goal was achieved at 12 MPa in *circa* 34 min. On the other hand, keeping the pressure constant at 6 MPa, inactivation was achieved at 35 or 45 °C in less than 25 or 12 min, respectively. These D_p values suggest a lower resistance of apple juice polyphenoloxidase than that reported in the literature. To this regard, Gui et al. (2007) reported a 220 min D_p value for polyphenoloxidase of cloudy juice from *Fuji* apples upon exposure at 35 °C to 30 MPa CO_2. These differences confirm the significant effect of processing conditions and *cultivar* on inactivation of apple polyphenoloxidase.

D_p values (Table 1) were used to calculate the parameter z_p, describing the sensitivity of polyphenoloxidase to pressurised CO_2. The decimal logarithmic values of D_p resulted well correlated ($R^2 > 0.89$; $p < 0.05$) with pressure for treatments carried out at 20, 35 and 45 °C (Table 2).

255 The z_p value, which represents the pressure range within which the D_p changes tenfold, resulted
256 *circa* 20 MPa for treatments carried out at 20 °C. This indicates that an increase in pressure of 20
257 MPa is necessary to get a 90 % decrease in D_p at this temperature. The increase in temperature from
258 20 to 45 °C did not cause a significant decrease in z_p value, indicating a constant effect of pressure
259 in inactivating the enzyme, at least within the temperature range here tested. To this regard,
260 contradictory data are reported in the literature. Weemaes et al. (1998) detected antagonistic effects
261 of pressure and temperature studying the effects of high static pressure combined with heating
262 treatments on avocado polyphenoloxidase. On the other hand, when conditions similar to those here
263 considered were applied on a plyphenoloxidase model system, a synergistic effect of pressure and
264 temperature was observed (Manzocco et al., 2016). Values of z_p (Table 2) thus emphasise the
265 critical role of enzyme origin and reaction media in determining its sensitivity to CO_2 pressure.
266 The effect of pressure on polyphenoloxidase inactivation was also expressed through the activation
267 volume ($\Delta V^{\neq}$) concept (Table 2). According to the transition state theory, the activation volume is a
268 measure of the volume difference between the initial reactants and the activated complex at the
269 transition state (Eyring, 1935). Data reported in Table 2 show that polyphenoloxidase inactivation is
270 characterized by negative $\Delta V^{\neq}$ with high absolute value (R^2 >0.89; p<0.05). This indicates that the
271 increase in pressure strongly favoured the denaturation of the enzymatic protein (Ohmae,
272 Murakami, Gekko & Kato, 2007). The values of activation volume for apple juice
273 polyphenoloxidase resulted lower than that reported in the literature (-94.3 cm^3 mol^{-1} at 55 °C; Gui
274 et al., 2007). This result indicates that, in our experimental conditions, polyphenoloxidase was more
275 susceptible to CO_2 pressure variation. In addition, in agreement with z_p values, activation volume
276 did not significantly decrease with the increase in temperature from 20 to 45 °C (Table 2). The
277 increase in temperature promoted instead a significant increase in the pre-exponential or frequency
278 factor ($ln\ k_{atm}$). The latter indicates how often the enzyme is properly oriented to undergo structural
279 modifications leading to denaturation. In fact, the increase in temperature from 20 to 45 °C

280 enhanced the frequency factor (Table 2), indicating a higher frequency of steric conditions
281 favouring denaturation.
282
283 *3.2. Effect of high pressure carbon dioxide on apple juice quality during storage*
284 In the light of the previous results, it can be hypothesized that HP-CO_2 treatment could represent a
285 non-thermal technological strategy to control enzymatic activity during storage of apple juice. In
286 this context, HP-CO_2 could be proposed as a technology to stabilize refrigerated apple juice colour.
287 To verify this hypothesis, a combination of processing conditions which could be potentially
288 applicable on a larger scale to produce fresh refrigerated apple juice was selected. To this regard,
289 treatments carried out at 20 °C were excluded since associated with residual activity higher than
290 30% even at the highest tested pressure (Table 1). The mildest pressure/temperature combination
291 leading to the minimum residual activity (20%) was thus selected. As shown in Table 1, this
292 combination corresponded to the treatment at 12 MPa and 35 °C. The juice was thus treated at these
293 conditions for 10 min since longer treatment times did not promote further enzyme inactivation
294 (Figure 1).
295 Even if similarly effective in terms of enzyme inactivation, treatments at temperature and pressure
296 higher than 35 °C and 12 MPa respectively, were not considered since they are reasonably more
297 energy-intensive and thus less sustainable from an environmental point of view.
298 Juice submitted to the selected treatment was then stored for up 15 days at 4 °C to simulate
299 conventional distribution conditions of not thermally stabilized apple juice.
300 During storage, apple juice was analyzed for the evolution of polyphenoloxidase activity and
301 browning. Microbial and sensory analyses were also performed to evaluate the hygienic level and
302 the intensity of typical sensory attributes of the juice. Data were compared to those relevant to an
303 untreated apple juice as well as a control thermal pasteurized apple juice (71 °C for 6 s). As
304 expected, the latter presented no enzymatic activity during the entire storage time, in agreement
305 with literature data (Golan-Goldhirsh, Whitaker & Kahn, 1984; McEvily, Iyengar & Otwell, 1992).

306 By contrast, HP-CO_2 treated and untreated apple juice showed different initial polyphenoloxidase
307 activity, which progressively decreased during storage, approaching in both cases 5% after 10 days
308 (Figure 2). These different inactivation degrees were probably associated with different evolution of
309 browning (Whitaker, 1995; Yoruk & Marshall, 2006).
310 For this reason, juice browning was assessed spectrophotometrically at 420 nm (Figure 3).
311 Immediately after preparation, untreated, pasteurized and HP-CO_2 treated apple juices showed not
312 significantly different browning. As expected, pasteurized juice did not show changes in browning
313 during storage, due to the complete and irreversible inactivation of polyphenoloxidase upon thermal
314 treatment. On the contrary, an increase in browning was detected in both untreated and HP-CO_2
315 treated samples. Beyond 3 days of storage, the latter showed browning values not significantly
316 lower than those of the untreated sample, suggesting that the HP-CO_2 treatment here applied was
317 not able to significantly reduce browning phenomena during storage.
318 To evaluate the ability of HP-CO_2 treatment to stabilize fresh apple juice against microbial spoilage,
319 total viable and lactic acid bacteria, yeasts and moulds were determined during storage. Whilst
320 pasteurized apple juice always presented microbial counts below the detection limit (data not
321 shown), untreated and HP-CO_2 treated apple juice showed different evolution of these microbial
322 populations during storage (Figure 4).
323 In the just prepared apple juice, lactic acid bacteria and moulds were below the detection limit and
324 the total bacterial count was mainly represented by yeasts. These microorganisms are well known to
325 be the main spoilage agents in fruit derivatives, due to their low pH (Raybaudi-Massilia et al.,
326 2009). During the refrigerated storage, total bacterial count of untreated juice progressively
327 increased up to 6 Log CFU/mL (Figure 4a) due to the growth of both yeasts and lactic acid bacteria
328 (Figures 4b and c). HP-CO_2 treatment allowed to decrease the initial count of total viable bacteria
329 and yeasts and to inhibit their growth during the storage (Figure 4a and 4b). In fact, after 15 days of
330 refrigerated storage, total bacteria and yeast resulted about 4 and 1 Log lower than that of the
331 untreated sample, respectively. On the contrary, lactic acid bacteria were below the detection limit

in HP-CO_2 treated juice, independently on storage time. Lactic acid bacteria have been actually reported to be more HP-CO_2 sensitive than yeasts (García-Gonzalez, Geeraerd, Elst, Van Ginneken, Van Impe & Devlieghere, 2009). As reported in the literature, the antimicrobial effects of HP-CO_2 are attributed not only to pressurization but also to media acidification (Balaban et al., 1991). To this regard, HP-CO_2 treatments were associated to a decrease in product pH due to the presence of residual carbonic acid after the treatment (Hong, Park & Pyun, 1997; Xu et al., 2011). However, in this study, the pH of the juice (4.2 ± 0.2) did not change upon the HP-CO_2 treatment and resulted analogous to that of pasteurized and untreated apple juice ($p>0.05$). It is thus likely that CO_2 residues were removed from the juice during the depressurization of the reactor after the treatment.

In order to evaluate the possible impact of HP-CO_2 treatment on sensory parameters of apple juice, the samples, stored for increasing time at 4 °C, were submitted to sensory evaluation. No significant changes in the evolution of the sensory attributes "acidity", "fresh apple flavour" and "sweetness" were detected by the panelists ($p>0.05$). In addition, judges did not detect any off-flavour in the samples. By contrast, significant changes in the scores of the sensory attributes "browning" and "cooked apple flavour" were noticed (Figure 5).

Due to complete polyphenoxidase inactivation, the browning sensory score of pasteurized sample resulted always significantly lower than that observed in untreated and HP-CO_2 treated juices, which showed progressively higher values (Figure 5a), mimicking the evolution of absorbance at 420 nm during storage (Figure 3).

Immediately after the treatment, the pasteurized juice presented a high "cooked-apple flavour" score, confirming the well-known sensory quality depletion induced by thermal treatment (Aguilar-Rosas, Ballinas-Casarrubias, Nevarez-Moorillon, Martín-Belloso & Ortega-Rivas, 2007). After few days of storage, the intensity of this defect in the pasteurized juice decreased, possibly because of the evolution of the juice sensory profile. On the contrary, judges were not able to detect this defect in the HP-CO_2 treated juice. The latter also presented mean "cooked-apple flavour" values comparable to those of the untreated juice during the entire storage period ($p>0.05$) (Figure 5b).

This result is certainly attributable to the low temperature (35 °C) experienced by the juice during the HP-CO_2 treatment.

4. Conclusions

HP-CO_2 treatments at temperatures lower than 45 °C may allow partial inactivation of polyphenoloxidase in apple juice. The treatment time needed for reaching the minimum residual activity decreases with pressure and temperature but no further inactivation is obtained by increasing pressure and temperature beyond 12 MPa and 35 °C respectively. HP-CO_2 treatment could be applied under mild pressure/temperature conditions for short times to allow a significant microbial stabilisation of fresh refrigerated apple juice without impairing its fresh-likelihood. Being HP-CO_2 treatment cheap and sustainable, these outcomes make it an interesting stabilisation technology for the production of fresh refrigerated apple juice.

Acknowledgement

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Table 1. Minimum residual activity (*RA%*) of polyphenoloxidase, apparent inactivation rate constants (*k*), relevant determination coefficient (R^2) and D_p values following HP-CO_2 treatments of apple juice at increasing pressures (0.1, 6.0, 12.0 and 18.0 MPa) at 20, 35 and 45 °C.

Temperature (°C)	**Pressure (MPa)**	**Minimum *RA%***	***k* (min^{-1})**	**R^2**	**D_p (min)**
20	0.1	69.0 ± 2.2 [a]	-0.0053 ± 0.0008 [b]	0.94	188.8 ± 26.8 [a]
	6	55.3 ± 1.0 [b]	-0.0207 ± 0.0024 [b]	0.97	48.4 ± 5.6 [bc]
	12	48.0 ± 1.8 [c]	-0.0297 ± 0.0053 [b]	0.94	33.7 ± 6.0 [bc]
	18	30.2 ± 1.5 [de]	-0.0452 ± 0.0063 [b]	0.96	22.1 ± 3.1 [c]
35	0.1	35.5 ± 0.6 [d]	-0.0133 ± 0.0019 [b]	0.94	75.1 ± 10.5 [b]
	6	24.6 ± 0.8 [e]	-0.0394 ± 0.0107 [b]	0.87	25.4 ± 6.9 [bc]
	12	18.0 ± 1.2 [f]	-0.0660 ± 0.0180 [b]	0.84	15.2 ± 4.1 [c]
	18	18.0 ± 0.6 [f]	-0.1228 ± 0.0530 [b]	0.97	8.1 ± 3.5 [c]
45	0.1	32.5 ± 1.3 [de]	-0.0465 ± 0.0055 [b]	0.97	21.5 ± 2.5 [c]
	6	19.0 ± 1.2 [f]	-0.0803 ± 0.0141 [b]	0.92	12.5 ± 2.2 [c]
	12	19.3 ± 0.8 [f]	-0.4107 ± 0.1034 [a]	0.89	2.4 ± 0.6 [c]
	18	20.3 ± 1.1 [f]	-0.6005 ± 0.1051 [a]	0.94	1.7 ± 0.3 [c]

Table 2: z_p (MPa), $\Delta V^{\neq}$ (cm^3 mol^{-1}) and *ln* k_{atm} (min^{-1}) values of apple juice polyphenoloxidase inactivation by HP-CO_2 treatments carried out at increasing temperature (20, 35 and 45 °C). Coefficients of determination (R^2) are also shown.

Temperature (°C)	**z_p (MPa)**	**$\Delta V^{\neq}$ (cm^3 mol^{-1})**	***ln* k_{atm} (min^{-1})**	**R^2**
20	20.3 ± 5.0 [a]	-276.7 ± 68.9 [a]	-4.94 ± 0.14 [b]	0.89
35	19.2 ± 2.4 [a]	-307.9 ± 38.4 [a]	-4.17 ± 0.17 [ab]	0.97
45	14.8 ± 2.6 [a]	-412.3 ± 73.8 [a]	-3.13 ± 0.31 [a]	0.94

Figure

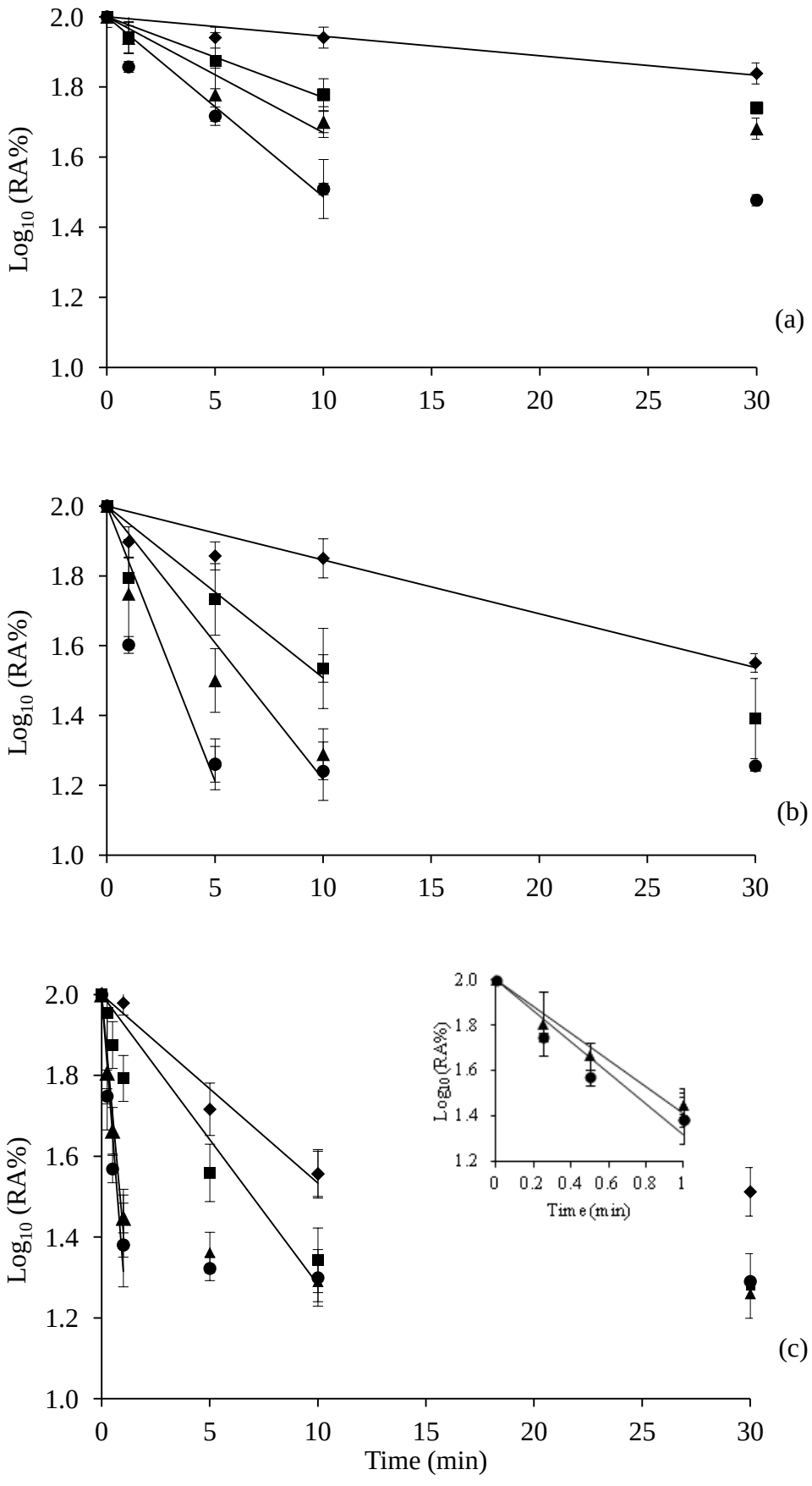

Figure 1: Polyphenoloxidase residual activity (*RA%*) of apple juice as a function of exposure time to increasing CO_2 pressures (6.0, 12.0 and 18.0 MPa) at 20 (a), 35 (b) and 45 °C (c). Samples treated at environmental pressure (0.1 MPa) were considered as control. Symbols: experimental data. Solid lines: regression lines obtained in the linear part of the curve. Inset of figure 1c: magnification of *RA%* in the 0-1 min time range.

Figure

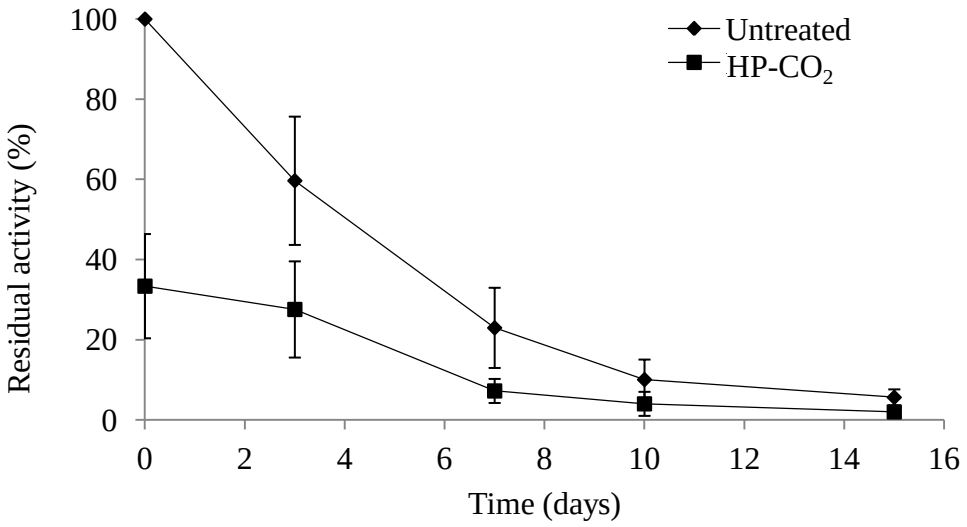

Figure 2. Polyphenoloxidase residual activity (%) during storage at 4 °C of HP-CO_2 treated and untreated apple juice.

Figure

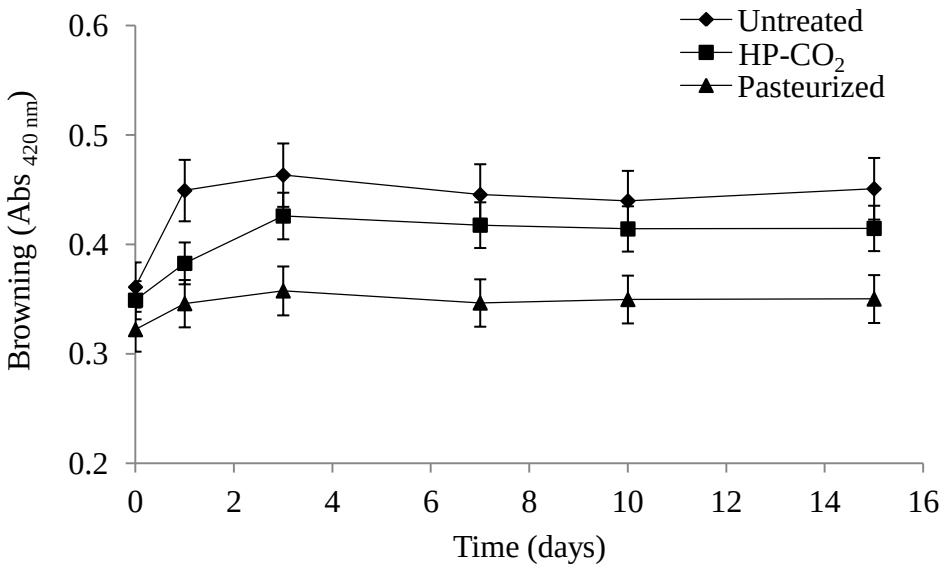

Figure 3. Absorbance at 420 nm of pasteurized, untreated and HP-CO_2 treated apple juices, during refrigerated storage.

Figure

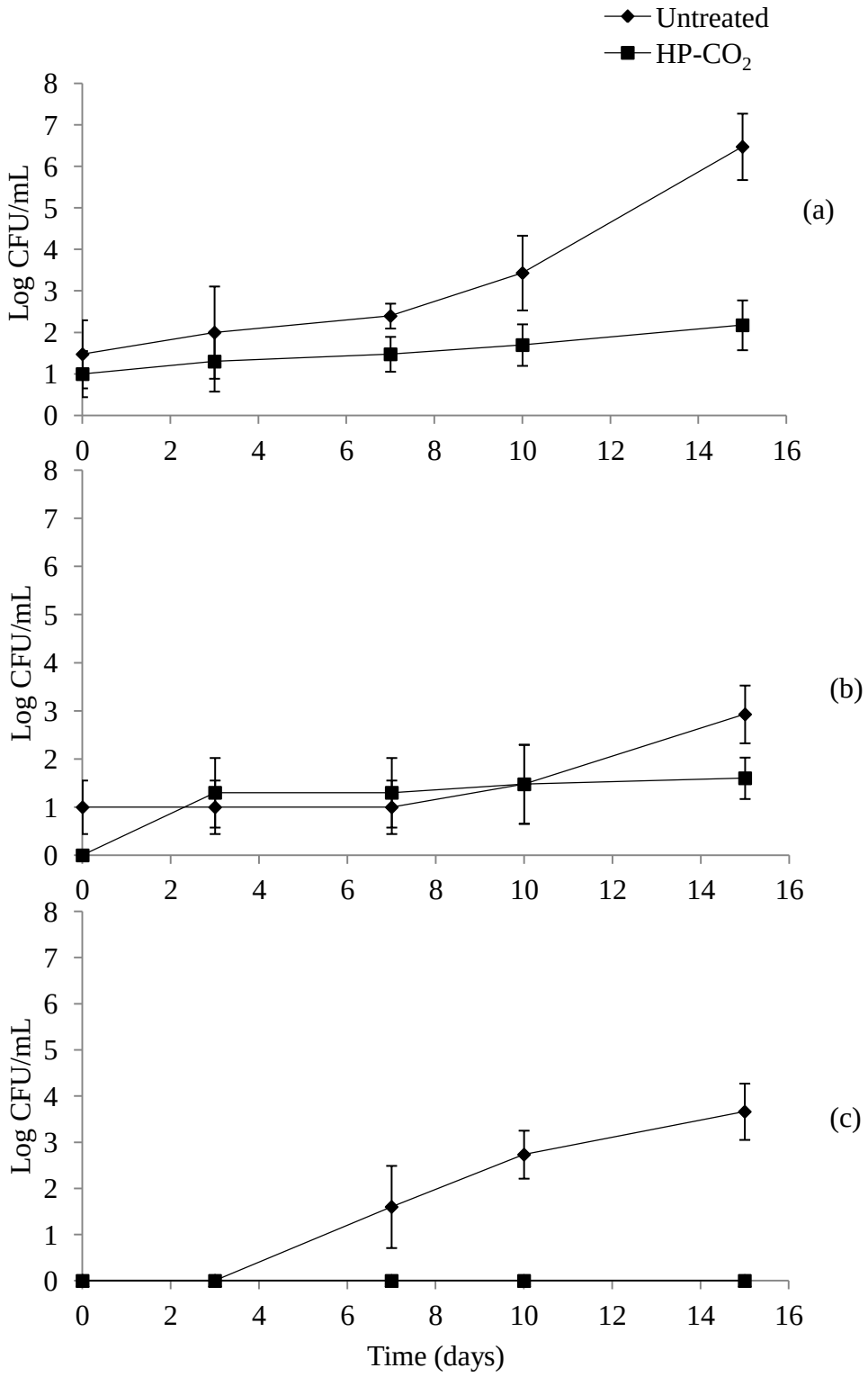

Figure 4. Total viable bacteria (a), yeast (b) and lactic acid bacteria (c) counts of untreated and HP-CO_2 treated apple juice, during refrigerated storage.

Total viable bacteria and yeast detection limit: 10 CFU/mL

Lactic acid bacteria detection limit: 1 CFU/mL

Figure

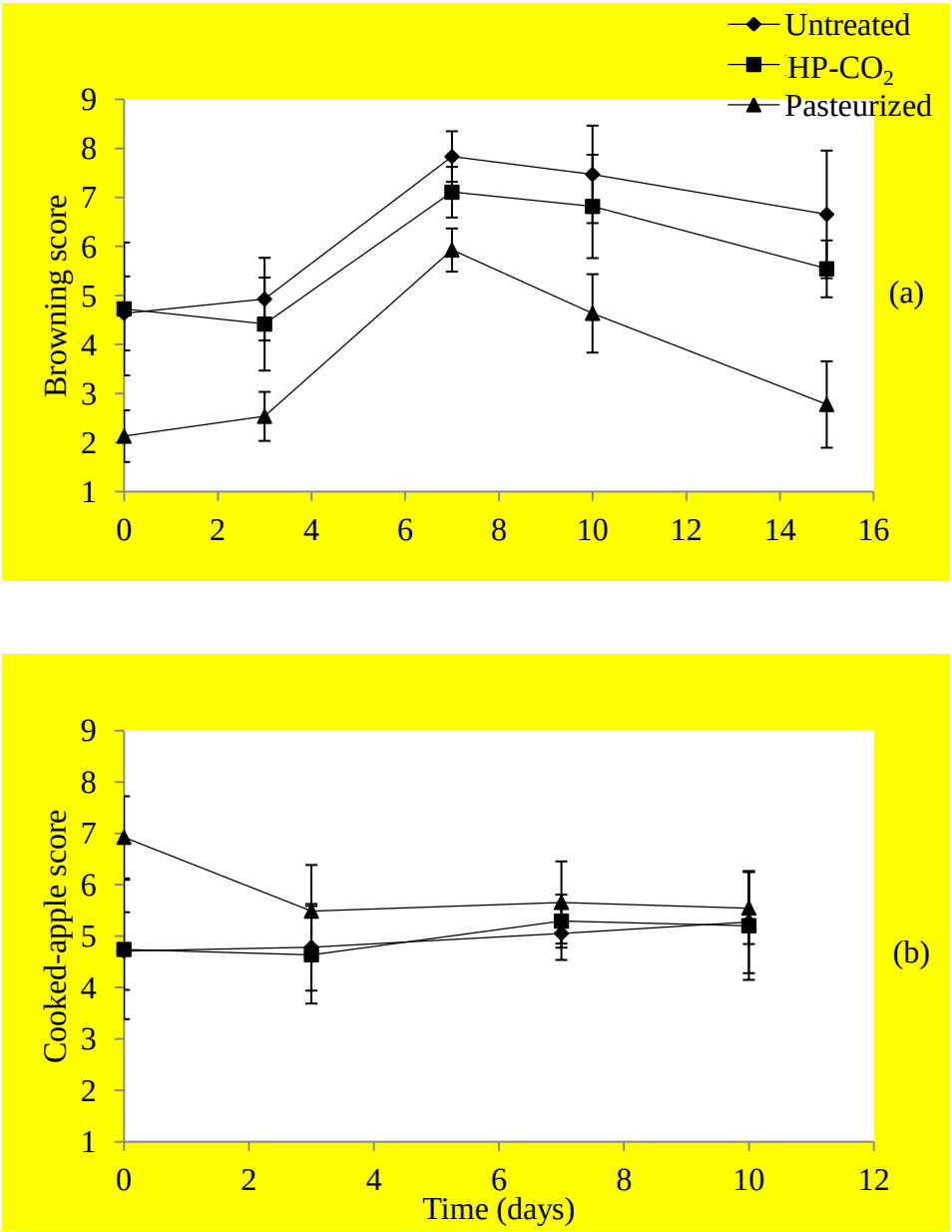

Figure 5. Browning (a) and cooked-apple flavour (b) sensory scores of pasteurized, untreated and HP-CO_2 treated apple juice, during refrigerated storage.